GLYCOGENESIS AND GLUCONEOGENESIS IN THE ISOLATED, PERFUSED RAT LIVER

Rudolph Jonathan Goerke III

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Acknowledgment . . .

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To My Wife

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INTRODUCTION

The purpose of this study is to propose a method for clarifying the roles played by the liver and circulating hormones in regulating mammalian carbohydrate metabolism: more particularly, for clarifying the manner in which insulin depresses blood glucose and cortisone-like compounds promote glycogenesis and gluconeogenesis.

These phenomena have been investigated by literally hundreds of competent researchers and yet no real agreement has been reached regarding the important mechanisms. Some (1, 2, 3, 4, 5, 6) feel that the liver's response to glucose levels and secondarily to hormone levels is the prime regulating factor, while others believe that the periferal effects of insulin and the adrenal corticoids (7) are more important. It is a truism that the construction of an experiment conditions the results, and certainly the great variety of findings is the simple consequence of a variety of experimental approaches.

In the following pages the isolated, perfused rat liver preparation is proposed as a means of establishing the relative importance of the various known control mechanisms. It is compared with some of the other more commonly used preparations and its particular advantages and limitations are described. The results of several experiments illustrating glycogenesis and gluconeogenesis are included with discussion; some modifications of the apparatus are suggested; and two further experiments are proposed.

CRITICAL REMARKS CONCERNING THE USE OF THE ISOLATED PERFUSED RAT LIVER IN METABOLIC STUDIES

Historically, the whole animal was the first preparation to be used in physiological experiments, although the natural attraction of isolated organ systems prompted investigation by many in the second half of the nineteenth century. This latter interest was eclipsed by Warburg's development of the more easily managed tissue slice techniques, and this form of experimenting has evolved through several lines into techniques using tissue homogenates and even cell-free systems consisting only of enzymes and substrates.

All of these techniques are necessary for gathering information on metabolism. In using the whole-animal preparation one can see any given biological change in context and thus obtain a picture of physiological relativity not present in the isolated enzyme system. Conversely, the whole-mammalian-organism is provided with so many adaptive mechanisms that primary metabolic changes are frequently difficult to discern, and simpler preparations become a necessity.

The isolated, perfused rat liver is situated, biologically speaking, halfway between the whole-animal and the liver slice. In this unique position it supplies some, but not all of the "compensating actions" of the entire body, and thus can be compared to the low power objective of the anatomist's microscope

which provides the necessary bridge between gross appearance and detailed structure.

The apparatus used in our studies is described in detail below. Suffice it to say here that it is a modification of those described by Brauer *et al*. (8) and Miller *et al*. (9), and consists of an isolated rat liver perfused via the portal vein with a heated, oxygenated, and recycled, diluted blood perfusate, which permits the addition and removal of perfusate and the removal of bile. Our preparation meets most of the requirements listed by D'Silva (10) for satisfactory isolated organ preparations.

Isolated organs generally present a myriad of technical difficulties in use, but many of these can be surmounted and the perfused specimen, in the case of the liver, retains many of those functions previously demonstrated *in vivo*.

A cursory glance through the literature will bear out this statement. As early as 1875 Luchsinger (11) observed the formation of glycogen in an artificially perfused liver, and preservation of this function has been described by a number of other observers (12, 13, 14, 15). Still others have demonstrated: gluconeogenesis (16, 17, 18, 19, 20); metabolism of fat (20); production of bile, RES activity, and BSP excretion (8); preservation of normal histology (8, 10); vasomotor reactivity (21); metabolism of corticosteroids (22, 23); synthesis of plasma proteins (9, 24); synthesis and degradation of cholesterol with maintenance of normal cholate perfusate level (25);

urea formation from ammonia (26); and the metabolism of acetate (27).

Besides retaining a great many physiolgical functions, the isolated liver possesses many advantages over the whole animal when used in biological experiments. Its use eliminates the metabolic competition of other tissues for substrates and also prevents the introduction of metabolic products from other tissues. The presence of hormones is much more adequately controlled, and it is particularly easy to introduce new variables into the perfusate and to withdraw large numbers of samples without compromising the integrity of the preparation.

It has still other advantages over the use of liver slices, homogenates, and extracts. First, it separates bile from the perfusate, a necessary function if the observations of Seckel (28) on cholate produced glycogenolysis are valid. Second, it preserves intact cellular boundaries which makes possible the use of a perfusate with extracellular composition and thus includes, in all biochemical reactions, the passage of substrates across these boundaries. Hastings and Flink (29, 30) have indicated that media approximating intracellular composition are necessary for valid slice work; and other researchers, among them Bowyer (31), Graubarth (32), Guest (33), Levine (34, 35), and their coworkers, have demonstrated the importance of intact cellular boundaries in evaluating metabolic systems. Third, the ease with which substances are added to and serial samples taken from the perfusate of the perfused liver is in contrast to the slice preparation in

which typically rather small volumes are used and volume changes must be limited.

There are many serious drawbacks to the use of the artificially perfused liver, however. Prime among the difficulties is that of providing adequate controls. Our own experience and that of D'Silva (10) indicate that removal of control lobes of the liver prior to perfusion results in many unsatisfactory preparations, and the adequacy of those livers that do survive is consequently open to question. Paired rats must therefore be used, and a fairly considerable variation of results is introduced.

The procedure itself is difficult, time consuming, and quite expensive. A fairly elaborate apparatus must be constructed and cleaned with each experiment; and many animals must be sacrificed to supply one liver with sufficient blood: one laboratory worker would be overworked if required to prepare and manage more than three experiments in a day's time. Also the nature of the apparatus requires that it be watched carefully throughout each run, lest an accident destroy all the work that had gone into its preparation.

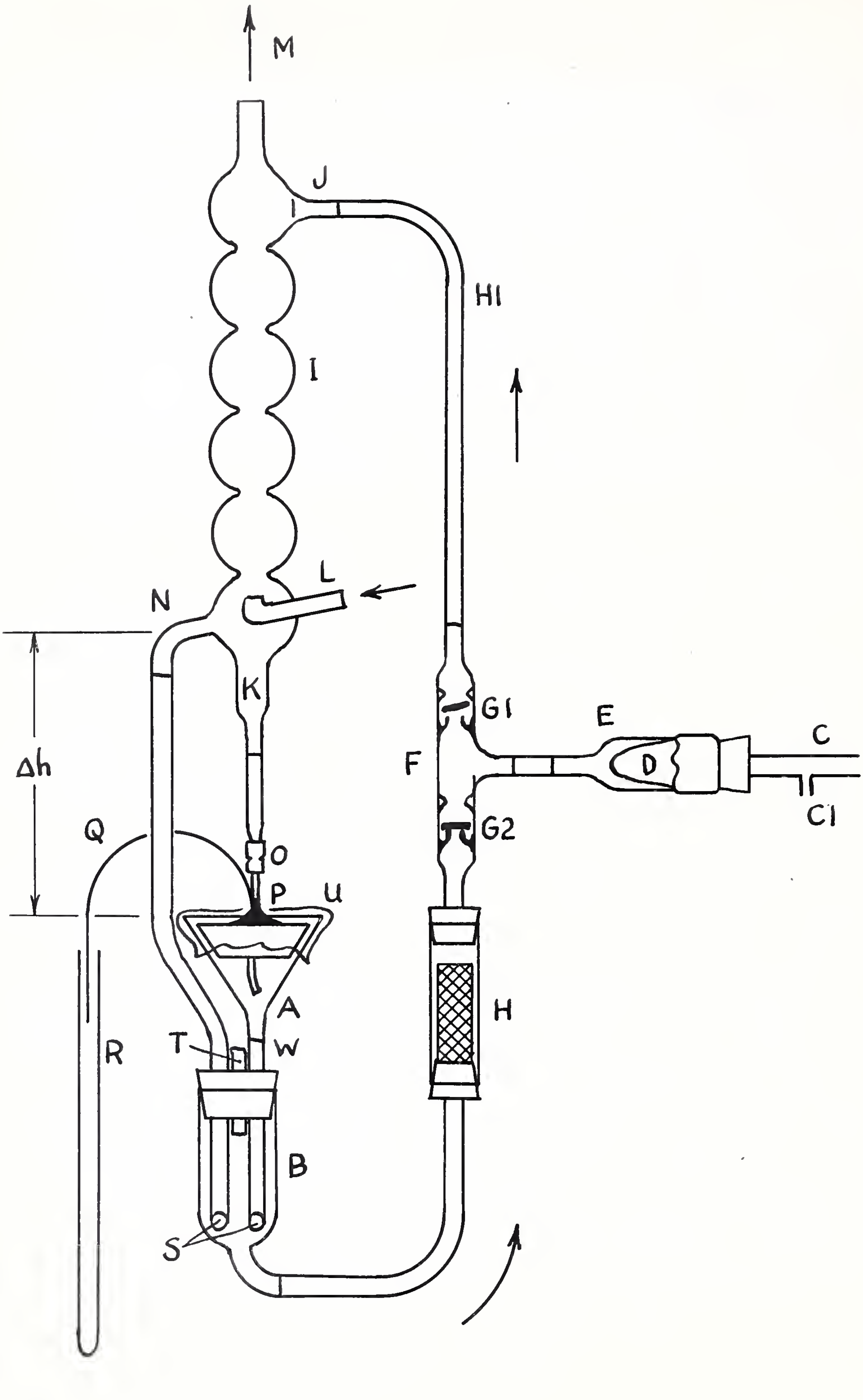
Beyond this there are difficulties inherent in our particular apparatus which might not be applicable to others. The introduction of blood cells offers a real complication as it provides a tissue to catabolize glucose and produce lactate at a rate which varies with ionic concentrations and pH values. If hemoglobin-Ringers solution is substituted for the blood-perfusate,

the liver is confronted with a vast load of hemoglobin which demands excretion: this could therefore not be used in studies of bile, and there is also a question as to whether interference would exist with other functions of the liver when it is required to excrete large amounts of bile pigments. The presence of a recycling perfusate, which is found in our preparation, slices and whole animals, means that constant levels of a given substrate are difficult to obtain except by very accurate injection techniques. Also, the possibility of contamination of the perfusate with hormones cannot be ruled out unless one assays the perfusate prior to each experiment: an almost impossible task.

Further difficulties center around the oxygenation problem: if a normal pH is to be preserved, then the perfusate must be in equilibrium with air or oxygen containing 5% carbon dioxide. But, this would render very difficult any study involving carbon dioxide measurement because of the great dilution of metabolically produced carbon dioxide by that contained in the oxygenating mixture.

One further difficulty, peculiar to experiments using Brauer's (8) perfusate, is the high content of buffer phosphate. This should tend to make glycogenesis difficult, according to the data of C.F. Cori et al. (36) who have shown that glucose-1-phosphate will not be incorporated into the glycogen molecule when the ratio glucose-1-phosphate/ $HPO_4^{=}$ is less than 0.4.

In summary, the isolated, perfused liver is situated biologically between the whole-animal preparation and the liver slice. It has been shown to preserve qualitatively many of the functions of the liver *in vivo*, and to possess many obvious advantages over liver slices on the one hand and the whole-animal on the other. Although it is a difficult preparation to manage, its unique advantages will command its use when the experimentor wishes to know how biochemical reactions work in context.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EXPERIMENTAL METHOD

Equipment and Materials

Perfusion Apparatus

The perfusion apparatus is a simplification of that described by Brauer et al (8), and uses an oxygenator described by Miller et al (9). All glass parts, with the exception of the oxygenator, have been coated with a silicone preparation (General Electric "Dry-Film 9987") and all tubing is of polyethylene. The entire apparatus is enclosed within a constant temperature cabinet held at 37° C.

The following is the description of a perfusion using the apparatus pictured above.

A measured amount of blood-perfusate, usually 100 ml., is poured into funnel (A) and flows into reservoir (B). An intermittent positive-pressure is applied at (C) by a respirator pump, and this pressure distends a rubber finger (D) inside pump (E) forcing air trapped behind the rubber finger into valve system (F) and past valve (G1). Air pressure keeps valve (G2) closed during this phase of the pump cycle. When the positive pressure of the respirator pump is no longer applied, the rubber finger is allowed to contract, forcing air out the side-arm (C1) and thus allowing outside atmospheric pressure to close valve (G1) and push part of the contents of the reservoir (B)

past Monel-metal filter (H) through valve (G2) into the pump system (E and F). Thus we have on the (C) side of rubber finger (D) a pulsating air pressure, and on the (E) side a pulsating perfusate pressure; hence further pulses will cause perfusate to enter the pump via valve (G2) and leave via valve (G1). The perfusate is thus caused to flow from pump (EF) up tube (H1) to the top of oxygenator (I), entering via tangentially placed port (J), and clinging in a thin film to the inside of the glass as it falls to the bottom of the oxygenator and into small reservoir (K). During its fall, the blood-perfusate equilibrates with the oxygen that is entering at (L) and flowing up through the oxygenator to leave at (M). Oxygen to be passed through the apparatus is first saturated with water vapor after being heated to 37^{o} C. This is accomplished by bubbling the oxygen through a large flask of water contained within the constant temperature cabinet. If this precaution were not taken the cool, dry oxygen would remove water from the perfusate and cool it below 37^{o} C. as it passed through the oxygenator.

Once in reservoir (K) the perfusate may either flow out the overflow (N) and back into reservoir (B) again, or it may enter the removable portal vein cannula (O) and thus enter the liver (P) placed in a small aluminum dish within funnel (A). Perfusate passing out the liver via its vena cava cannula is drained through funnel (A) back into reservoir (B).

The bile duct cannula (Q) carries bile from liver (P) to bile

collecting tube (R) which is calibrated in hundreths of a milliliter.

Perfusate entering the reservoir (B) does so through tubes under the fluid surface in the reservoir in order to minimize foaming. Baffles (S) at the ends of these tubes assure mixing in the reservoir. A vent (T) loosely plugged with cotton allows the withdrawal of samples and also equilibration with outside air pressure. A plastic cover (U) seals off the funnel (A) and prevents drying of the surface of the liver.

Perfusate time rate of flow is measured by pinching shut the outflow tube of the funnel at (W) and measuring the time required to fill the funnel to a known volume represented by a mark etched on the glass.

Constant infusions were added to the perfusate in some of our experiments by means of a fine polyethylene tube entering through the oxygen port (L) and ending within the portal vein cannula (O).

Materials for the Perfusion Apparatus

Polyethylene tubing (Clay-Adams Co.):

- 0.038" O.D. x 0.023" I.D., bile duct cannula
- 0.095" O.D. x 0.066" I.D., portal vein and vena cava cannulas
- 0.290" O.D. x 0.250" I.D., perfusate tubing

Oxygenator, valve system, and reservoir

(Blown to order of pyrex glass by MacAlaster-Bicknell Inc., New Haven, Conn.)

Glass funnel (approximately 50 mm.) "siliconed"

Rubber finger from rubber glove

Rubber finger chamber from drip chamber of standard glass blood infusion set as used in hospitals.

Monel-metal filter from same blood infusion set.

Filter housing made of "siliconed" glass tubing.

Rubber stoppers to fit reservoir and rubber finger chamber

Aluminum dish for liver (milk bottle cap)

Respirator pump (capable of at least 60 cycles per min.)

Constant temperature cabinet (4 x 4 x 1½ feet) with transparent front and mounting rods

Perfusion Solutions

Perfusing solutions consisted of whole rats' blood 40% and 60% of either Solution A or Solution B, described in detail below. Sprague Dawley male rats weighing from 250 to 450 grams and not starved were used as blood donors. The animals were bled from their aortas under ether anesthesia using #20 gauge "siliconed" needles, and "siliconed" syringes with from 9 to 17 ml. being obtained from each rat. The blood was Heparinized as it was drawn by prior rinsing of the syringes with a Heparin solution containing 300 units per milliliter of isotonic saline. The drawn pooled blood was stored up to three days in polyethylene bottles under refrigeration.

1000 ml. of Solution A were prepared as follows: 5587 mg. NaCl, 187 mg. KCl, and 314 mg. $CaCl_2.2H_2O$ were mixed in approximately 700 ml. distilled water. To this mixture was added 40.0 gm. bovine plasma albumin (Fraction V, Armour & Co.) which was also mixed well. Then 100.0 ml. 0.100M Na_2HPO_4, 20.0 ml. 0.100M KH_2PO_4, and a solution of 2268 mg. $NaHCO_3$ in approximately 100 ml. of distilled water were added slowly with stirring, after which the volume was made up to 1000 ml. with distilled water. If the chemicals were added in the above order, there was no precipitate of insoluble calcium salts. When mixed as above solution had a pH of 7.4 and the following ionic concentrations in mEq./L.: Na^+ 142.5, K^+ 4.5, Ca^{++} 5.0, Cl^- 103.0, HCO_3^- 27.0, and

$HPO_4^=$ 22.0. It follows that the protein concentration was 4.0%. In practice amounts of 1 L. were not prepared at one time, but only amounts sufficient for four perfusions. Terramycin (Pfizer), 5 mg./100 ml. of solution, was added just prior to use for bacteriostasis; and Anti-foam A (Dow-Corning) was sprayed for two seconds on the surface of the perfusate at the same time to suppress foaming during circulation. Solution A was used in all cases unless otherwise noted.

1000 ml. of Solution B was prepared in the same manner with the following exceptions: 1850 mg. $NaHCO_3$ and 15.0 ml. 0.100M Na_2HPO_4 replaced previously used amounts, and 22.0 ml. of 1.00M Na r-Lactate (Eli Lilly & Co) was added. This modification replaced about one fifth of the bicarbonate and four fifths of the phosphate with racemic lactate and did not modify the cation concentrations, or the pH.

In two experiments, LP #30 and #31, 18.6 mg. of powdered Compound F - alcohol (Schering Corp.) was added to the 93 ml. of perfusate initially present in each case and allowed to equilibrate during hepatectomy, after the method of Miller *et al*. (9).

In one experiment, LP #32, 30 mg. of Compound F - alcohol were dissolved in 7.5 ml. of 50% ethanol in isotonic saline, and 4.0 ml. of this solution (ie: 16 mg. Cpd. F) injected at a constant rate over a three hour period into the portal vein cannula. In another experiment, LP #33, an equivalent amount of ethanol was injected.

Methods

Operative Procedure

Sprague Dawley male rats weighing from 250 to 400 grams and starved from 22 to 30 hours prior to operation were used as liver donors, except where otherwise noted.

Just before each operation the perfusion machine was filled with the measured mixed perfusate which was then allowed to circulate and equilibrate at 37°C. with oxygen. In experiments #34 through #39, a mixture of 95% oxygen and 5% carbon dioxide was used in place of the oxygen. Average time for operation, and therefore equilibration, was 30 minutes.

Using ether anesthesia and clean but non-sterile technique, laparotomy was performed and a loose ligature placed around the inferior vena cava just above the entrance of the right renal vein. 0.2 ml. of a heparin solution containing 500 units per ml. of isotonic saline were injected with a #27 needle into the inferior vena cava below the ligature.

Following cannulation of the bile duct the gastrosplenic ligament was torn and the small lobe of the liver situated behind the stomach freed of ligamentous attachments. Double ligatures were tied around the esophagus including the left gastric artery, and both these structures were cut between the ties thus mobilizing the stomach.

Two small lobes of the liver were then ligated and removed

for glycogen analysis. This last procedure was omitted after LP #34 when it was found to result in mechanical obstruction of either the portal vein or the bile duct in about ½ of the operations, and livers from paired rats were then used as glycogen controls. At this time a sample of the perfusate was taken for analysis.

Then a loose tie was placed around the portal vein just after the entrance of the splenic vein, care being taken to include the hepatic artery and to exclude the bile duct. A traction ligature was tied about the portal vein approximately 3 cm. caudad to the first tie, and the vein cannulated, using the first ligature to secure it. The period of time elapsing between the tying of the portal vein and the perfusion of the liver was recorded in each case; it averaged 8 minutes.

After the anterior chest wall was removed, both phrenic nerves sectioned to immobilize the diaphragm, and a ligature tied around the inferior vena cava just below the atrium, the vena cava was cannulated above the diaphragm. Next the loose tie about the inferior vena cava below the liver was tightened, the remaining blood in the thoracic and abdominal cavities sponged away for better visualization, and the liver was removed as rapidly as possible to the perfusion machine. Just prior to being fitted to the machine, the portal vein cannula was filled with 0.9% NaCl from a needle and syringe in order to expel any air bubbles that might otherwise have passed to the liver and obstructed its blood supply. Extreme care was taken not to chill the liver during any of these procedures.

Once the liver had been positioned for maximum perfusate flow, the free end of the bile duct cannula was inserted into the bile measuring tube and the liver — containing funnel was covered with a transparent plastic cover.

After LP #34 the initial samples of perfusate were obtained 5 to 10 minutes after perfusion started, so that added blood from the liver would be included in the analyses. Since the pump output is greater than 100 ml. per min. and the volume of perfusate in the system about 100 ml., this time should have been adequate for mixing.

All perfusions were run for 3 hours and final volumes determined after the livers had been removed for glycogen analysis. Final perfusate samples were taken in all cases, and in several experiments samples were taken at 90 minutes with appropriate correction in the calculated data for the substances removed.

Chemical Analyses

Glucose: Blood-perfusate glucose was determined according to the method of Nelson (37) as modified by Somogyi (38).

Glycogen: Liver glycogen was determined according to the method of Good, Kramer, and Somogyi (39) with determination of the resultant glucose by the method cited above.

Urea: Blood-perfusate urea was determined by the xanthydrol method of Engel and Engel (40) on tungstate filtrates.

Lactate: Plasma-perfusate lactate was determined according to the method of Barker and Summerson (41) on trichloroacetic acid filtrates.

TABLE 1

GROUP A

LP #	LIVER WT. IN MG.	PERFUSATE GLUCOSE			LIVER GLYCOGEN			PERFUSATE UREA			3 HR. BILE IN ML.
		INITIAL IN MG./100 ML.	3 HR. IN MG./100 ML.	3 HR. Δ MG. CORR.	INITIAL IN %	3 HR. IN %	3 HR. Δ MG.	INITIAL IN MG./100 ML.	3 HR. IN MG./100 ML.	3 HR. Δ MG. N CORR.	
36	3830	45	95	+39	0.152	0.001	-5.5	35.8	55.9	+2.3	0.47
22	6350	264	240	-38	0.034	0.067	+2.3				0.61
23	7690	268	212	-50	0.031	0.075	+3.4				0.51
38	8990	360	372	-9	0.057	0.322	+23.8	31.0	60.5	+4.6	0.72
39	7310	384	336	-55	0.057	0.438	+27.8	28.5	57.0	+4.7	0.33

EXPERIMENTAL RESULTS

Glycogenesis

Group A - LP #36, 22, 23, 38, and 39

Method:

In LP #36 female liver and blood donors were used, and in LP #38 and 39 female liver and blood donors of a hooded strain of rats were used; otherwise procedures were as indicated above in experimental method.

Results (Table #1):

In LP #36 with an initial perfusate glucose level of 45 mg./100 ml. the perfused liver lost 5.5 mg. of glycogen during the 3 hour period. In LP #22 and 23 with an average glucose level of 266 mg./100 ml. the livers were seen to gain a small amount of glycogen, while in LP #38 and 39 at an average glucose level of 372 mg./100 ml. an average increase of 25.8 mg. of glycogen was noted.

GROUP B

	PERFUSATE GLUCOSE		
LP #	INITIAL IN MG. / 100 ML.	3 HR. IN MG. / 100 ML	3 HR. ΔMG. CORR.
26	172	176	0
27	172	188	+ 2
28	236	164	-59
29	244	172	-58
24	296	208	-75
25	312	200	-93
37	414	408	-4

TABLE 2

Gluconeogenesis

Effect of Varying Perfusate Glucose

Group B - LP #26, 27, 28, 29, 24, 25, 37, 36, 19, 21, 20, 18, 17
22, 23, 38, and 39

Method:

LP #36, 38, and 39 were modified as in Group A above. LP #26 and 27 were experiments in which Solution A without blood was circulated for 3 hours with determinations of initial and final glucose levels. In LP #28, 29, 24, 25, and 37 a mixture of blood and Solution A was circulated with the same determinations being done. In LP #21 and 20 female liver donors were used; otherwise procedures used were those described in Experimental Method above.

Results (Table #2):

In the two bloodless perfusions, LP #26 and 27, there was essentially no change in the glucose level of the perfusate over a three hour period. In LP #28, 29, 24, and 25, with pure oxygen being supplied to the blood there was drop in the perfusate glucose which was greater as the initial glucose level was increased. In LP #37, in which 5% CO_2 was included with the oxygen, there was essentially no change in the glucose level even with a very high initial level.

(Over)

GROUP B - CONT'

LP#	LIVER WT. IN MG.	PERFUSATE GLUCOSE INITIAL IN MG./100 ML.	PERFUSATE GLUCOSE 3 HR. IN MG./100 ML.	PERFUSATE GLUCOSE 3 HR. Δ MG. CORR.	LIVER GLYCOGEN INITIAL IN %	LIVER GLYCOGEN 3 HR. IN %	LIVER GLYCOGEN 3 HR. Δ MG.	PERFUSATE UREA INITIAL IN MG./100 ML.	PERFUSATE UREA 3 HR. IN MG./100 ML.	PERFUSATE UREA 3 HR. Δ MG. N CORR.	3 HR. BILE IN ML.
36	3830	45	95	+39	0.152	0.001	-5.5	35.8	55.9	+2.3	0.47
19	7200	56	102	+53	0.021	0.036	+1.1				0.98
21	4930	66	128	+50	0.017	0.013	-0.2				0
20	4830	68	128	+47	0.016	0.041	+1.2				0.30
18	6880	76	114	+38	0.033	0.055	+1.5				0
17	9020	82	138	+61	0.032	0.074	+3.7				1.14
22	6350	264	240	-38	0.034	0.067	+2.3				0.61
23	7690	268	212	-50	0.031	0.075	+3.4				0.51
38	8990	360	372	-9	0.057	0.322	+23.8	31.0	60.5	+4.6	0.72
39	7310	384	336	-55	0.057	0.438	+27.8	28.5	57.0	+4.7	0.33

TABLE 3

Results (Table #3):

In these experiments, arranged in the table according to increasing perfusate glucose level, there is seen a transition from gluconeogenesis to glucose disappearance. Liver glycogen content does not vary significantly except in LP #38 and 39, in which there is a large amount of glycogen formed and also a large discrepancy between values for glucose disappearance.

TABLE 4

GROUP C

LP #	LIVER WT. IN MG.	PERFUSATE GLUCOSE			LIVER GLYCOGEN			3 HR. BILE IN ML.
		INITIAL IN MG./100 ML.	3 HR. IN MG./100 ML.	3 HR. ΔMG. CORR.	INITIAL IN %	3 HR. IN %	3 HR. ΔMG.	
22	6350	264	240	-38	0.034	0.067	+2.3	0.61
23	7690	268	212	-50	0.031	0.075	+3.4	0.51
33[a]	9180	276	259	-17	0.015	0.063	+4.4	0.83
30[b]	9840	248	256	-22	0.050	0.119	+6.8	1.44
31[b]	7290	244	260	+3	0.083	0.086	+0.2	0.78
32[b]	8980	266	245	-20	0.011	0.036	+2.3	1.27

a - PERFUSATE CONTAINS ALCOHOL

b - " " CPD. F

Effect of Hydrocortisone

Group C - LP #22, 23, 33, 30, 31, and 32

Method:

Livers and blood were obtained in the usual manner. LP #22 and 23 are included as control experiments since their initial perfusate glucose levels are comparable to the experimentals. LP #33 was <u>in</u>fused with ethanol, powdered Hydrocortisone was added to LP #30 and 31, and LP #32 was infused with Hydrocortisone: details of these procedures are described under Experimental Method above.

Results (Table #4):

In LP #22 and 23 an average of 44 mg. of glucose disappeared from the perfusate, although only 17 mg. of glucose disappeared from the perfusate into which ethanol was infused. In the experiments to which Hydrocortisone was added the average disappearance of glucose was 15 mg., however, the variation was large. The average 3 hour bile flow without hormone was 0.65 ml. and with hormone was about twice this value, or 1.16 ml.

GROUP D

TABLE 5

LP#	LIVER WT. IN MG.	PERFUSATE GLUCOSE			LIVER GLYCOGEN			PERFUSATE ...			3 HR. BILE IN ML.
		INITIAL IN MG./100 ML.	3 HR. IN MG./100 ML.	3 HR. ΔMG. CORR.	INITIAL IN %	3 HR. IN %	3 HR. ΔMG.	INITIAL IN MG./100 ML.	3 HR. IN MG./100 ML.	3 HR. ΔMG. CORR.	
								... LACTATE			
28		236	164	-59				59.2	107.0	+35.1	
29		244	172	-58				62.9	106.0	+32.5	
30^a	9840	248	256	-22	0.050	0.119	+6.8	42.0	46.2	-1.6	1.44
31^a	7290	244	260	+3	0.083	0.086	+0.2	38.0	40.0	0	0.78
								...UREA		...UREA N	
34^b	10380	168	248	+67	0.068	0.082	+1.5	24.4	53.3	+5.6	0.56
35^b	7810	180	272	+72	0.152	0.095	-4.5	24.9	46.0	+4.2	0.55

a - PERFUSATE CONTAINS CPD. F

b - " " ADDED LACTATE

Effect of Perfusate Lactate Level

Group D - LP #28, 29, 30, 31, 34 and 35

Method:

In LP #34 and 35 hooded male rats were used as liver and blood donors. LP #28 and 29 used circulating blood-perfusate but no liver, while Hydrocortisone was added to the perfusates of LP #30 and 31; these last four experiments are included because it was possible here to obtain initial and final perfusate lactate levels. In LP #34 and 35 Solution B was used in place of Solution A; this would yield a lactate concentration of 13.2 mM/L., or 118 mg./100 ml., in the blood-perfusate assuming that the blood compartment contributed no lactate.

Results (Table 5):

In LP #28 and 29 an average increase of 38.3 mg. of lactate was noted, while in LP #30 and 31, with the addition of livers, there was a negligible change in perfusate lactate. In the liverless experiments there was a relatively large decrease in perfusate glucose, while in those containing livers there was an equivocal change. LP #34 and 35 showed a marked increase in average perfusate glucose; unfortunately it was not possible to obtain lactate determinations on these perfusates. If one assumes, however, that the amount of lactate added to the blood-perfusate by the blood was the same as the average in the foregoing experiments, then the initial perfusate lactate level would have been about

TABLE 6

GROUP E

		PERFUSATE GLUCOSE			LIVER GLYCOGEN			PERFUSATE UREA			
LP#	LIVER WT. IN MG.	INITIAL IN MG./100 ML.	3 HR. IN MG./100 ML.	3 HR. Δ MG. CORR.	INITIAL IN %	3 HR. IN %	3 HR. ΔMG.	INITIAL IN MG./100 ML.	3 HR. IN MG./100 ML.	3 HR. Δ MG. N CORR.	3 HR. BILE IN ML.
37		414	408	−4				19.8	21.0	+0.2	
34	10380	168	248	+67	0.068	0.082	+1.5	24.4	53.3	+5.6	0.56
35	7810	180	272	+72	0.152	0.095	−4.5	24.9	46.0	+4.2	0.55
36	3830	45	95	+39	0.152	0.001	−5.5	35.8	55.9	+2.3	0.47
38	8990	360	372	−9	0.057	0.322	+23.8	31.0	60.5	+4.6	0.72
39	7310	384	336	−55	0.057	0.438	+27.8	28.5	57.0	+4.7	0.33

165 mg./100 ml. in LP #34 and 35.

Urea Production

Group E - LP #37, 34, 35, 36, 38, and 39

Method:

In LP #37 no liver was included in the circuit. In LP #34 and 35 hooded male rats were used as liver and blood donors. In LP #36 Sprague-Dawley females were used for liver and blood, and in LP #38 and 39 hooded females were used as liver and blood donors. Initial and final perfusate urea determinations were done in all cases.

Results (Table #6):

LP #37 without a liver showed no change in perfusate urea nitrogen content. The remaining experiments, however, all showed an increase in perfusate urea nitrogen content and at the same time demonstrated a wide variation in glucose change. If micrograms urea nitrogen production per gram of wet liver per three hours is calculated, the following values are obtained: LP #34 (539), LP #35 (538),LP #36 (601), LP #38 (512), and LP #39 (643).

DISCUSSION OF EXPERIMENTS

Glycogenesis

It is evident from the experiments in Group A that glycogen formation is a function of perfusate glucose level. The data is not sufficient to warrant calculation of equilibrium constants, but it is seen that a greater than five-fold increase in liver glycogen content occurred in the two experiments whose average initial perfusate glucose level was 372 mg./100 ml. At the other end of the scale, the perfusion with an initial glucose concentration of 45 mg./100 ml. was seen to cause a decrease in liver glycogen. This conclusion agrees with the findings of other observers who obtained liver glycogen formation only when the perfusate glucose level was 602 to 908 (13), 1500 (14), 2000 (11), or 5000mg./100 ml. (15).

The effect on glycogenesis of Hydrocortisone was investigated in Group C, and it was seen that even though more than 16 mg. of Hydrocortisone was supplied the liver along with an average glucose level of 253 mg./100 ml. there were insignificant increases in liver glycogen. Corey _et_ _al_. (15) however have shown that with glucose concentrations of 5%, the isolated liver was capable of manufacturing glycogen when the perfusate contained 5% of adrenal cortical extract (Schering).

It is concluded from the data that an increase in glucose concentration above 372 mg./100 ml. will cause deposition of

glycogen in the isolated perfused liver. It cannot be said whether this mechanism is of importance under physiological conditions.

Gluconeogenesis

Groups B,C, and D above bear upon gluconeogenesis. In group B, much the same situation applied as did with glycogen formation, that is: an increase in initial perfusate glucose concentration depressed gluconeogenesis (at the same time as it would seem to have increased glycogenesis). This phenomenon was investigated in 10 experiments whose strictly quantitative comparison is prevented because of the presence or absence of carbon dioxide in the oxygenating gases, and variations in animal strains used.

The effect of the administration of Hydrocortisone upon gluconeogenesis was obscured somewhat by a rather large experimental variation, however, the data indicates that the hormone potentiated gluconeogenesis in our preparation.

In Group D raising the perfusate lactate level was seen to produce a marked rise in gluconeogenesis, presumably from the lactate itself.

These findings agree well with the established mechanisms of glucose formation by the liver. Glycogenolysis was observed inadvertantly in one of our preliminary preparations in which the liver donor had managed to obtain food during his supposed 24 hour fast: liver glycogen fell from 2.7 to 0.1% while perfusate glucose rose from 75 to 200 mg./100 ml. in the 3 hour period.

An uncontrolled error in these experiments lies in the failure to determine lactates in all occasions. Since lactate accumulates on storage of blood and storage varied from 1 to 3 days this might introduce serious errors.

It is therefore concluded that perfusate glucose level alone is a sufficient stimulus for the gluconeogenic maintenance of perfusate glucose level by the isolated liver, in accord with some whole-animal experiments (2, 3). Hormonal and lactate modificiation of this stimulus seems indicated.

Urea Production

Group E is discussed separately from gluconeogenesis because the data indicate no correlation between formation of glucose and urea formation. Urea nitrogen production in micrograms of urea per gram of wet liver per three hours averaged 566 ± 24* . At the same time glucose production per three hours varied from --55 to + 72 mg. Whether or not this lack of correlation represents failure of gluconeogenesis from protein breakdown could better be determined by analysis of perfusate ammonium content, since urea production from ammonium may be a limiting factor here.

* SE of mean $= \sqrt{\dfrac{\Sigma(y-\bar{y})^2}{N(N-1)}}$

Bile Production

An interesting by-product of the experiments in Group C was the fact that added Hydrocortisone caused an increase in bile production. Increased bile was also noted in several preliminary experiments using adrenal cortical extract. This phenomenon has been noted in bile fistula animals (42).

Glucose Utilization by Circulating Blood Cells

It was found in Group B that if the liver was not present and if CO_2 was not used in the oxygenating gases then the blood cells would remove more glucose from the system as the initial glucose concentration was increased. At the highest concentration of glucose, however, CO_2 was supplied, and a negligible amount of glucose was found to have disappeared from the perfusate. This finding is in accord with those of Long (43) and Graubarth *et al.* (32) who demonstrated inhibition of glycolysis in acidotic blood. It also demonstrates the necessity of maintaining a proper concentration of CO_2 in the oxygenating gas when the isolated liver is used in carbohydrate studies.

INDICATIONS FOR FURTHER STUDY

Modifications of the Technique

As noted above there are a number of disadvantages associated with the isolated liver preparation as it was used in these experiments. Certain of these disadvantages are amenable to change and the following modifications are suggested.

First among the obligatory changes is the removal of most of the phosphate-buffer compartment so as not to depress abnormally the formation of glycogen. If some 2.0 mEq./L of phosphate were retained the remainder need not be replaced, since the buffering actions of hemoglobin, other proteins, and bicarbonate should be sufficient over the course of any experiment. Since the protein (bovine plasma albumin) has been supplied in all probability as the sodium (and potassium) salt, the ionic concentrations as put forth in Experimental Method above represent plasma minus protein salt, and hence the actual concentrations after protein addition are probably too high in sodium and potassium (as well as phosphate). It follows that removal of sodium and potassium phosphates will tend to correct an ionic imbalance rather than create one.

Next among the necessary modifications is the introduction of hemoglobin and hematocrit determinations and red blood cell count to provide control checks on hemolysis and on the mass of this circulating-metabolizing tissue. Concurrent use of dye such

as T1824 would provide another check on volume changes.

Another modification should be the inclusion of gas flowmeters and thermometers in the oxygenator circuit and at least two determinations during each experiment of both pCO_2 and pH. The thermometer would give visual proof that saturated gases were being warmed to 37° C. and hence were not removing water vapor from the system. Flowmeters would assure reproducible amounts of CO_2 being removed from the perfusates. The determinations of pCO_2 and pH are necessary in view of the varying cellular metabolic rates associated with changes in pH that were mentioned above in the Discussion.

The problem of lactate levels deserves particular mention. It is suggested that serial lactates be done in several experiments in order to determine the earliest time after the start of perfusion at which "initial" samples should be obtained. Since the lactate level of blood increases during storage, this factor should either be eliminated by uniform storage times or accurately measured by lactate determinations. This, too, would influence the time of "initial" sampling.

An investigation of the glycogenetic effect of raising potassium levels may not be necessary, but it would certainly influence the composition of future perfusates and give much information regarding the integrity of cell walls in the preparation.

Further study of control problems is indicated: the removal of lobes from the liver after beginning perfusion might be found

to compromise perfusate flow less than lobe removal at the time of the initial operation.

Still another variable that bears investigation is the anti-foaming agent. The choice of the present compound was purely by chance, and it might be found that some of the newer synthetic detergents or other anti-foaming compounds were superior. In any case, toxicity should be determined.

It is also suggested that in experiments utilizing hormones, such as the adrenal steroids, that the hormones be infused over the length of the experimental period following a priming dose. Evidence of Louchart and Jailer (43) and others indicates that such compounds are rapidly rendered inactive by the liver.

Suggested Experiments

The following two schemes seem to offer a reasonable return on the investment of experimental effort.

Making use of a CO_2 - O_2 oxygenating mixture, a low perfusate phosphate, and lactate determinations of initial sampling times as outlined above, an experiment to test the glycogenetic ability of livers from normal versus livers from adrenalectomized rats can be easily designed. If perfusions are done two at a time with glucose levels of 375 mg./100 ml. and the perfusates allowed to mix with one another until the time of initial sampling, then a) livers from normal rats could be compared with those from adrenalectomized rats and b) livers from

adrenalectomized rats could also be compared with those later infused with Hydrocortisone. Six or more sets of infusions using paired normal and paired adrenalectomized rats for liver glycogen controls should give meaningful results. It is anticipated from the liver slice findings of Teng et al. (44) that livers from adrenalectomized donors would form more glycogen, but that hydrocortisone would depress glycogenesis.

Another experiment is suggested in which the liver's ability to manufacture glucose from extra-lactate sources can be tested. This could be accomplished by labeling the initial glucose pool with radioactive glucose and measuring its subsequent dilution by newly formed glucose. By using a range of initial glucose concentrations one could test the finding above that gluconeogenesis decreases in the face of increasing perfusate glucose concentrations.

SUMMARY

1. The isolated, perfused rat liver is presented as a difficult to prepare but biologically active preparation that is particularly useful in determining how biochemical reactions work in physiological context.

2. The method of preparing experiments is described, with a description of the apparatus and perfusion solutions.

3. Several experiments are recorded demonstrating glycogenesis, gluconeogenesis, and urea production. The data indicates that high glucose levels in the perfusate enhanced glycogenesis and depressed gluconeogenesis. High lactate levels and hydrocortisone administration produced gluconeogenesis, while the use of adrenal steroids in general was associated with an increased bile output from the livers. Urea production was relatively constant at varying rates of gluconeogenesis.

4. Suggestions are included for modifications of the technique and two promising experiments are outlined.

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